

The effect of Ganoderic Acid A on miR-17-5p and miR-181b expression level and apoptosis induction in human leukemia Nalm-6 cells

Faezeh Mortazavie MSc¹, Simin Taheri MSc¹, Parisa Tandel MSc¹, Farahnaz Zare MSc¹, Gholmossein Tamaddon PhD^{1*}

1. Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran.

*Corresponding author: Dr Gholmossein Tamaddon, Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran. Email: tamaddon.g@gmail.com. ORCID ID: 0000-0001-8158-6004

Received: 27 September 2021

Accepted: 11 May 2022

Abstract

Background: In various cancers, Ganoderic Acid A (GAA), an active triterpenoid derived from *Ganoderma lucidum*, has been proved to show potent anti-tumor effects. However, the possible impacts of GAA on the human leukemia cell line (Nalm-6) are not fully elucidated. Therefore, this research aimed to study the antineoplastic effect of GAA on Nalm-6 cells.

Materials and Methods: In this laboratory trial study, Nalm6 cells were cultured in vitro and treated with different doses of GAA (25, 50, 100, 200, and 400 µg/mL) for 24, 48, and 72 hours. The optimal treatment concentration of GAA was determined by the MTT assay. Flow cytometry was used to determine the death of Nalm-6 cells caused by GAA treatment by utilizing FITC-conjugated propidium iodide (PI) and annexin V staining. After incubation, the expression levels of miR-17-5p and miR-181b were monitored using real-time polymerase chain reaction (PCR).

Results: Based on the half-maximal inhibitory concentration (IC₅₀) measurements of the MTT assay, the optimal treatment concentration of GAA was 140 µg/mL (in a dose and time-dependent manner, $p < 0.0001$). The GAA treatment was selectively toxic to the leukemia Nalm-6 cells and could remarkably induce cell apoptosis ($p < 0.0001$). Besides, GAA downregulated the expression of miR-17-5p and miR-181b in the Nalm-6 cells compared with the untreated cells ($P = 0.0067$ and $P = 0.0014$, respectively).

Conclusions: Based on the present findings, GAA merits further investigation as a promising natural reagent for treating hematologic malignancies.

Keywords: Apoptosis, Ganoderic Acid A, MiR-17-5p, MiR-181b, Nalm-6 cells

Introduction

Acute lymphoblastic leukemia (ALL) is a hematologic neoplastic disorder and the most common pediatric cancer (0-14 years) worldwide (1). This hematologic malignancy accounts for approximately one-third of all childhood cancers (2). Despite various available treatments for ALL patients, these treatments are associated with a relatively high rate of side effects (3, 4). Moreover, resistance to conventional therapies and disease relapse can threaten ALL patients and lead to therapy delay or discontinuation (5). Therapy-related toxicity has become a major therapeutic challenge in ALL (6).

Therefore, developing novel and effective natural agents with minimum adverse effects to increase therapeutic efficacy is fundamental in ALL therapy. *Ganoderma lucidum* (*G. lucidum*), also known as "Reishi" and "Lingzhi" mushroom in Japan and China, respectively, has been consumed broadly as a health tonic and ancient therapeutic in China and other oriental countries (7). This beneficial fungus has been utilized to treat various conditions, including cancers, due to its significant salutary effects (8). Although different bioactive molecules have been identified in this medicinal mushroom, its

pharmacological activity is primarily attributed to polysaccharides and triterpenoids (9). Ganoderic Acid A (GAA) is the most promising candidate from the triterpenoid family, isolated from *G. lucidum* with a range of biological activities (10). Previous studies have shown that GAA exhibits various beneficial activities, such as antioxidative (11), anti-viral activity (12), hepatoprotective (13), and particularly anticancer activities (14). Currently, evidence suggests that GAA has remarkable cytotoxic effects against leukemia, lymphoma (15), hepatocellular carcinoma (16), osteosarcoma (17), and breast cancer cells (18) through suppressing cell growth and invasion and inducing apoptosis. Besides, GAA can be considered an adjuvant therapeutic intervention for various malignancies. MicroRNAs (miRNAs) are non-coding endogenous RNAs, ranging from 19 to 25 nucleotides in size, which modulate gene expression at the post-transcriptional level (19). Emerging evidence suggests that aberrant miRNA expression is correlated with multiple types of human diseases, including cancer (20). Considering the predominant function of miRNAs in cancers, they can act as either oncogenes or tumor suppressors by regulating their respective target genes (21). MicroRNA-17-5p belongs to the miR-17-92 cluster family, with an established oncogenic function (22). It is currently known that miR-17-5p is highly expressed in many human cancers, including ALL (23). It has been shown that miR-17-92 components lead to the progression of B-cell malignancies through downregulation of phosphatase and tensin homolog (PTEN) and enhancement of ACT/mTOR signaling pathway activation (24). As a member of the miR-181 family, miR-181b may have a unique role depending on tumor type and cellular environment (25-28). The literature suggests that the expression of miR-181b is directly regulated by signal transducer and activator of transcription

(STAT3) pathway and high-mobility group AT-hook 1 (HMGA) ((29). Notably, growing evidence shows that natural compounds can govern miRNAs, including the *G. lucidum* extract (30). Recently, it has been reported that *G. lucidum* polysaccharide extract can effectively inhibit the progression of hepatocellular carcinoma via downregulation of the accumulation of regulatory T cells by inducing miR-125b (31). Moreover, Qing-Ping Wu's study (2012) revealed that ergosterol peroxide obtained from *G. lucidum* could be used to counteract the drug resistance given by miR-378 (32). Additionally, Ganoderma lucidum polysaccharide extract inhibited Oral squamous cell carcinoma (OSCC) cell proliferation and migration by controlling the miR-188/BCL9/-catenin signaling system (30). According to the previous studies, Ganoderma lucidum extract could alter the expression of miRNAs in different cancer cell lines. Previous studies have shown that miR-17-5p and miR-181b act as incomers in the Nalm-6 cell line (33). Consequently, the present study was focused on miR-17-5p and miR-181b. This study investigated the impact of GAA content of the Chinese herb *G. lucidum* on apoptosis induction and alteration of miR-17-5p and miR-181b expression in Nalm-6 cells.

Materials and Methods

Materials

The American Type Culture Collection provided us with the Nalm6 B-cell precursor leukemia cell line (ATCC; Gaithersburg, Maryland, USA. Gibco Life Technologies (Waltham, MA) and Sigma-Aldrich (Munich, Germany) provided all of the ingredients and reagents utilized in cell culture. The cells were regularly maintained at 37°C in a 5 percent CO₂ and 95 percent air humidified environment in RPMI-1640 media supplemented with 10 percent fetal bovine serum (FBS), 2 mM l-glutamine, and 100 U/mL penicillin-streptomycin solution

(Sigma-Aldrich). Cell integrity was ensured by modifying the growth medium for the Nalm-6 cell line using conventional cell culture methods. Cell viability was also assessed using the trypan blue exclusion technique. The GAA extract was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and stock solution was made in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/mL and kept at -80°C.

MTT assay

The MTT test was used to determine the inhibitory effect of GAA on the metabolic activity of the cells. In a 96-well plate, 2×10^4 cells were added to 200 μ L of growth media and cultured for 24, 48, and 72 hours, using various doses of GAA (25, 50, 100, 200, and 400 μ g/mL). Next, 20 μ L of freshly prepared MTT solution (5 mg/mL in PBS) was added to every well then incubated at 37°C in a 5 percent CO₂ environment. The plate was incubated for 3-4 hours at 37°C before being centrifuged at 350 g for 10 minutes to remove the supernatant. Besides, 150 μ L of DMSO was used to dissolve the formazan crystals, and a BioTek ELx800 microplate photometer was used to examine the spectrometric absorbance at 570 nm (SN211805; BioTek, Winooski, VT, USA). The medication concentration that prevented cell growth by 50% was known as the half-maximum inhibitory concentration (IC₅₀). Each experiment was repeated three times to ensure accuracy.

RNA extraction

The TRIzol reagent (Thermo Fisher Scientific, MA, USA) was used for cellular RNA extraction in each group. An RNA spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, USA) was used to measure the amount and quality of RNA, which was then frozen at -80°C.

cDNA synthesis

The reverse transcriptase (RT) microRNA PCR kit bought from Pars Gene Company (Tehran, Iran) was used to synthesize cDNA using 2 μ g of total RNA for the

study. The RT enzyme was inactivated in all samples by heating the samples to 85°C for one minute. All samples were then incubated at 42°C for 60 minutes. The miR-17 and miR-181b qRT-PCR assays were performed using an ABI 7500 real-time PCR equipment (Applied Biosystems, Foster City, CA, USA), SYBER Green Master Mix (Pars Gene Co., Iran), and particular primers (Pars Gene Co., Iran). Primers sequences for Real-time PCR are presented in Table1. To normalize the levels of expression of each miRNA, the U6 rRNA gene was utilized as an internal reference.

Real-time PCR

During a typical PCR, denaturation, annealing, and extension cycles are repeated to achieve exponential amplification of the target sequence. Denaturation consists of robust heating of the samples to separate or denature the DNA strands, which provides a single-stranded template for the next step. Once the strands are separated, the temperature is decreased to the annealing temperature so that the primers may pair (or anneal) to complementary regions of the template. The polymerase extends the primer to form a nascent DNA strand during the extension step. The following conditions were used in the qRT-PCR assay: first, denaturation at 95°C for 15 minutes, then 40 cycles of 95°C for 30 seconds followed by 62°C for 30 seconds, and 72 °C for 30 seconds. The length of Real-time PCR products for miR-181b and miR-17-5p were 25 and 22 nucleotides, respectively. The comparative CT technique was used to determine the relative fold alterations for each sample ($RQ=2^{-\Delta\Delta C_t}$). The current study utilized target Scan, Miranda, and miRTarBase databases to find miRNAs and their target genes.

Flow cytometry

A kit developed by BD Biosciences (San Jose, CA) was utilized to measure the percentage of apoptotic cells following GAA treatment. In brief, Nalm-6 cells were collected 48 hours after being treated

with the specified dose of GAA isolated from *G.lucidum*. Phosphate buffered saline (PBS) was used to wash the cells two times before 5×10^5 of them were resuspended in 1 mL of 1X Annexin V binding buffer. The cells were then incubated in the dark for 20 minutes at room temperature with 5 μ L of fluorochrome-conjugated Annexin V and 5 μ L of PI staining solution. Next, 400 μ L of binding buffer was added to the cell suspension, centrifuged for five minutes at 400 g. FACS Calibur flow cytometer identified the Annexin V-positive/PI-negative as early phase apoptotic cells, whereas those positive for both Annexin V and PI were believed to be in the late stages of apoptosis or necrosis (BD Biosciences, USA). FlowJo software was used to analyze the data for the apoptotic population (TreeStar LLC, USA).

Ethical Consideration

The local ethics committee approved this experimental study of medical experiments of Shiraz University of Medical Sciences with the ethics code of IR.SUMS.REC1399.838.

Statistical analysis

The results are presented as the mean and standard deviation (SD) of three separate studies. GraphPad Prism was used to perform all analyses (GraphPad Software Inc. La Jolla, CA, USA). One-way variance analysis (ANOVA) was used to compare various groups. $P < 0.05$ was utilized as the statistical significance threshold.

Results

Analysis of the effect of GAA on cell viability in the Nalm-6 cell line

Chemical Structure of Ganoderic Acid A is shown in Figure 1. The findings showed that GAA significantly reduced the ability of Nalm-6 cells to proliferate. As shown in Figure.2, an MTT assay was used to determine the survival rate of Nalm-6 cells after 24, 48, and 72 hours of GAA treatment with deferent concentrations (25, 50, 100, 200, and 400 μ g/mL). Results of cell viability determination exhibited that

cell viability decreased in a dose-dependent and time-dependent manner. The IC₅₀ was used to establish the optimum GAA concentration. Based on the IC₅₀ measurements using MTT assay, the Nalm-6 cells could hardly grow in the presence of 140 μ g/mL of GAA for 48 hours ($p < 0.0001$). The present study's findings demonstrated the anti-proliferative effects of GAA content from *G. lucidum* on the human Nalm-6 cancer cells. Note that 140 g/mL of GAA was utilized in the subsequent tests after 48 hours.

Effect of GAA on cell apoptosis

The Annexin V-FITC Apoptosis Detection Kit was utilized to measure the amount of apoptosis based on the presence of phosphatidylserine in the outer membrane of Nalm-6 cells treated with *G. lucidum* GAA extract. Figure.3 illustrates the results of the experiment. Moreover, the Nalm-6 cells were exposed to GAA at a 140 g/mL concentration for 48 hours. The present findings of the present showed that the cells treated with Nalm-6 had a much higher proportion of apoptotic cells than the control groups. The present study's findings showed 40.5% of early and late apoptosis after 48 hours ($p < 0.0001$).

Effect of GAA on miR-17 and miR-181b expression in the Nalm-6 cell line

In the present study, to the best of our knowledge, qRT-PCR was performed for the first time for evaluating the expression of miR-17-5p and miR-181b in the Nalm6 cell line before and after GAA treatment. Figure.4 depicts the results of a miRNA RT-PCR experiment to see if GAA treatment (140 μ g/mL) for 48 hours affected the miR-17-5p and miR-181-b in expression Nalm-6 cells. It was demonstrated that miR-17 (median fold change [FC]=0.23; $P=0.0067$) and mir-181b (FC=0.01; $P=0.0014$) expression was significantly reduced in treated Nalm-6 cells compared to untreated cells according to the findings of Real-time PCR.

Also, results of the present study showed reduced miR-17-5p and miR-181b expression in the Nalm-6 cells treated with GAA compared to the group treated with L-asparaginase (P=0.042 and P=0.0053, respectively).

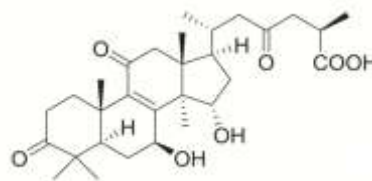


Figure1. Chemical Structure of Ganoderic Acid A

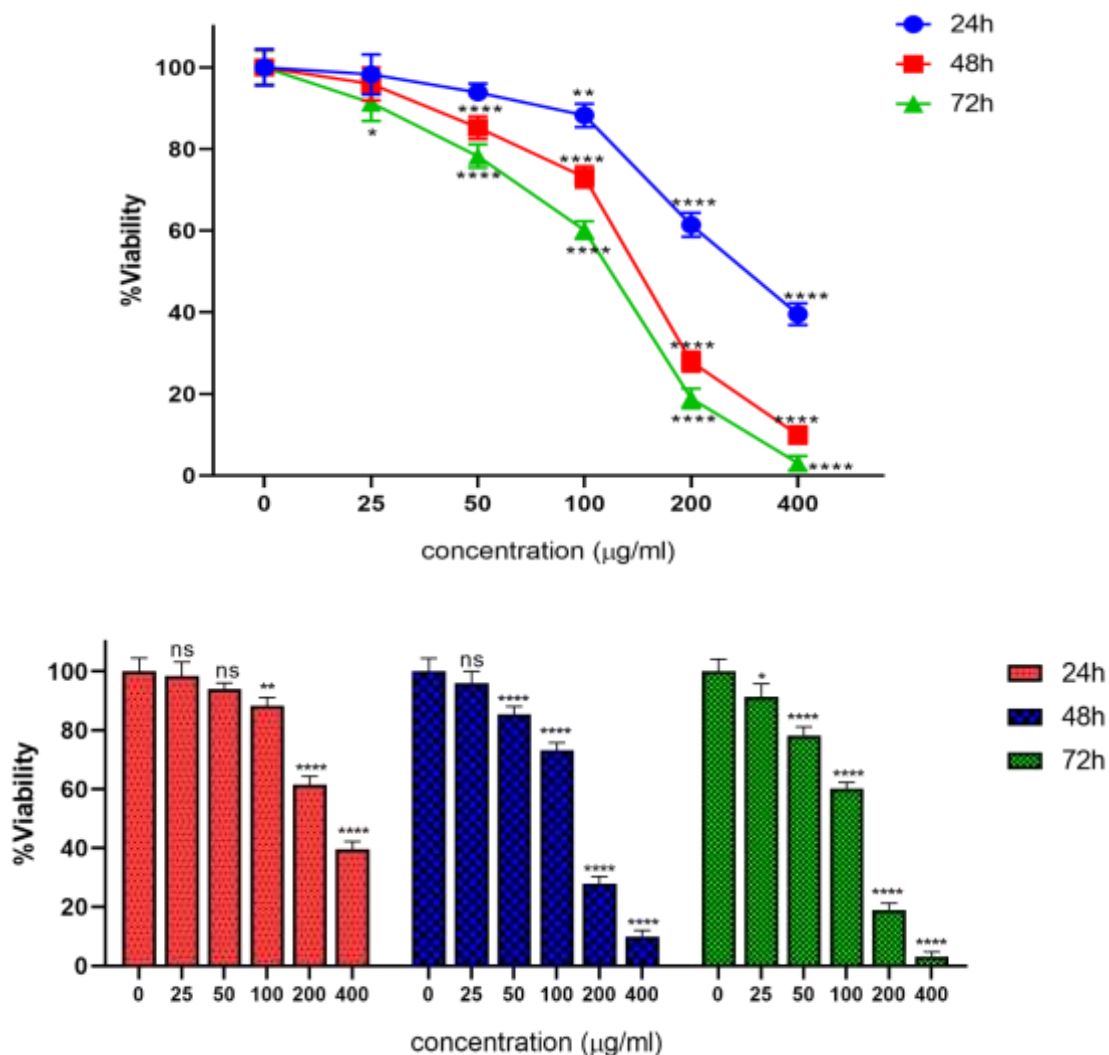


Figure 2. The IC₅₀ of GAA treatment in the Nalm-6 cells was determined using MTT assay. The Nalm-6 cells (2×10^4 cells/well) were plated into 96-well plates and treated with increasing concentrations (25, 50, 100, 200, and 400 µg/mL) of GAA for 24, 48, and 72 hours. Data are shown as mean \pm SD in triplicate. The cells treated without GAA were used as the controls.

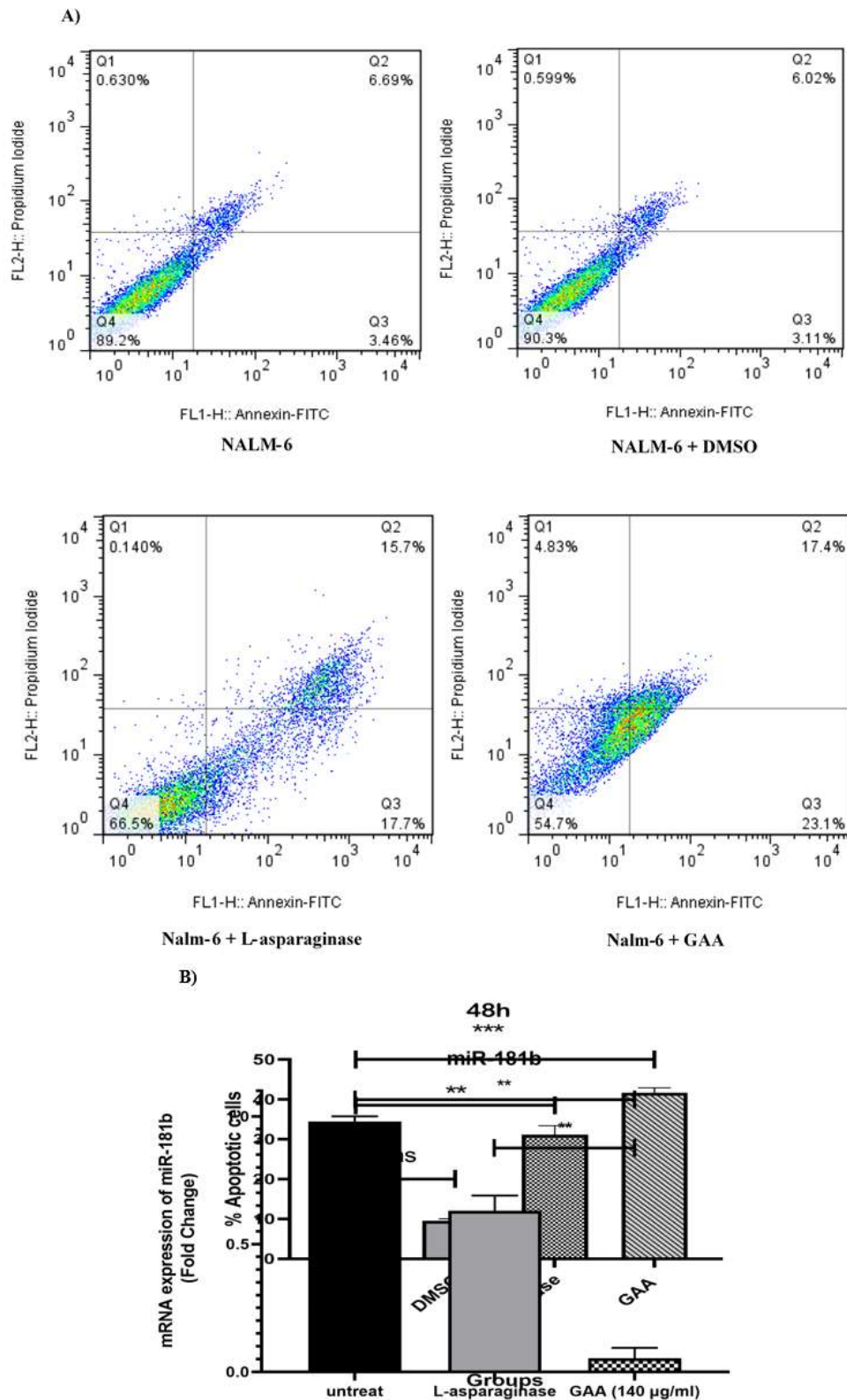


Figure 3. The effects of GAA on the apoptosis of Nalm-6 compared with the control groups. This diagram shows the Nalm-6 cells treated with 140 $\mu\text{g}/\text{mL}$ of GAA extract for 48 hours. Data are presented as mean \pm SD of at least three independent experiments. The apoptotic index is the sum of the percentage of positive cells for Annexin-V-FITC alone and the positive cells for both Annexin-V-FITC and PI ($p < 0.0001$).

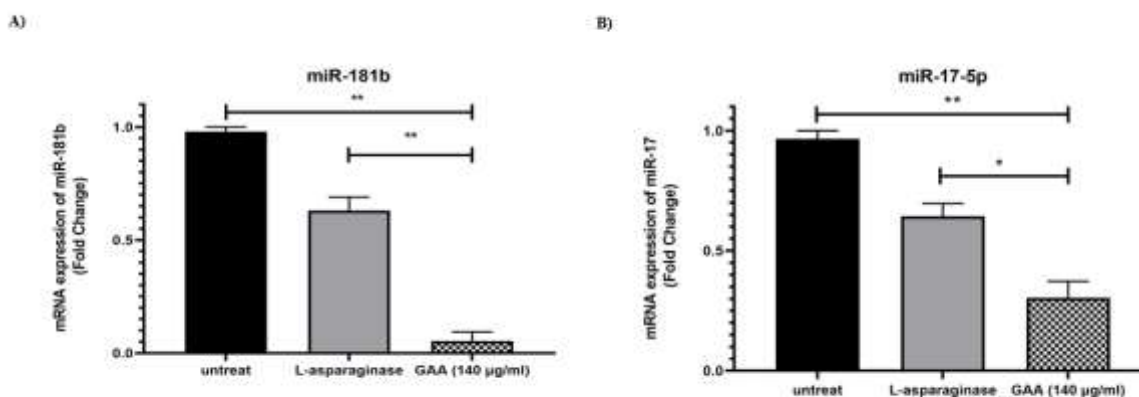


Figure 4. The expression levels of miR-17-5p and miR-181b in the Nalm-6 cells after treatment with GAA (140 µg/mL) for 48 hours. Significant differences in the expression levels of miR-181b (A) and miR-17-5p (B) were reported between the treated Nalm-6 cells and control groups by qRT-PCR assay. The U6 rRNA gene was used as the internal control (error bars represent standard deviations).

Discussion

GAA is a novel, highly oxidized triterpenoid extracted from the Chinese herbal medicine *G. lucidum*, which Asian physicians and naturopaths recommend preventing and treating various human diseases, including cancers (34). Substantial evidence suggests that GAA possesses significant anticancer properties against different human cancer cells in vitro (15-18, 35). Nevertheless, to the best of our knowledge, the molecular mechanism of GAA in the human cell line derived from B cell leukemia (Nalm-6 cell line) remains unknown, and there are only several pieces of literature of laboratory and preclinical studies on the anti-tumor activity of GAA in the Nalm-6 cells. Therefore, in the present study, the biological function of GAA was evaluated in the Nalm-6 cells in vitro and explored the potential underlying mechanisms associated with this activity. The present study showed that GAA treatment was cytotoxic and markedly suppressed cell proliferation and induced apoptosis in the Nalm-6 cells in vitro. Consistent with our findings, previous studies also established that GAA, as a component of GL extract, has cytotoxic effects on different cancer

cell lines, such as leukemia cell lines (15). More importantly, the study results revealed that GAA significantly downregulated the expression levels of oncomirs 17-5p and 181b in the Nalm-6 cells compared to the control groups. Substantial evidence suggests that natural products, including *G. lucidum* extract, exert their anti-tumor activities via several mechanisms, including miRNA expression modulation (36). Recently, it has been suggested that *G. lucidum* polysaccharides (GLPs) markedly inhibit hepatocellular carcinoma growth in vitro and in vivo by decreasing Treg cell accumulation, which is correlated with the upregulation of miR-125b (31). Besides, another study reported that ergosterol peroxide obtained from *G. Lucidum* exhibits profound activities in inducing the apoptosis of miR-378-transfected cells, overcoming resistance to multiple drugs (32). In addition, it has been demonstrated that GLP increased miR-188 in HSC-3 cells expression, and miR-188 blocked the activation of the β -catenin signaling pathway via the binding to BCL9 (37). The present study appears to be the first to examine the impact of GAA on miRNA expression. Therefore, it was attempted to determine whether miRNAs could be involved in the response

of treated Nalm-6 cells. Notably, previous studies have reported that miR-181b in multiple types of cancers, such as pediatric ALL, acts as an oncogene and dramatically leads to the growth and development of malignancies (38-41). Previous studies have shown STAT3 and HMGA1 directly modulate the expression of miR-181b (42, 43). Recent studies have shown that STAT3 is an oncogenic protein (44). The STAT3 has been linked to the development, progression, and invasion of various malignancies, including lymphoma, multiple myeloma, and leukemia. Interestingly, it has been reported that GAA inhibits the STAT3 signaling pathway and is highly susceptible to cisplatin-induced cell death in HepG2 cells (45). Additionally, a study on a breast cancer cell line found that GAA exerted anticancer effects through JAK2/STAT3 signaling pathway, and the activities of JAK2 and STAT3 were directly inhibited by GAA treatment (46). Moreover, Balraj Singh Gill et al. investigated the effect of GAA extract on human prostate cancer cells and reported that GAA inhibited cell proliferation and viability and downregulated the expression of STAT3 in prostate cancer (PC) cells (47). However, this study did not investigate the direct relationship between GAA and STAT3 inhibition. It can be postulated that significant downregulation of miR-181b expression by GAA is probably linked to GAA's ability to suppress the STAT3 pathway, which ultimately resulted in the death of Nalm-6 cells; accordingly, further investigation is needed in this field. Upregulation of the miR-17-92 cluster is a major oncogenic event in various cancer types, including breast cancer, prostate cancer, pancreatic cancer, colon cancer, lung cancer, lymphoma, and leukemia (48). The literature suggests that the miR-17-92 cluster interferes with the tumor-suppressive pathway and spreads malignancies (49). Recent studies have reported that a principal target of miR-17-

5p is the tumor suppressor, PTEN (24). It seems that the miR-17-92 components play a crucial role in the occurrence of B-cell malignancies by downregulation of PTEN potentiating the ACT/mTOR pathway (50). The PI3K/ACT/mTOR pathway is a substantial regulator of biological processes, including cell proliferation, survival, differentiation, death, cancer, and angiogenesis (51). The aberrant activation of at least one component of the PI3K/ACT/mTOR signaling network is correlated with the occurrence and development of tumors, including leukemia (52). Recent experimental findings suggested that human glioblastoma cells were strongly inhibited under GAA treatment via the PI3K/ACT signaling pathway inactivation (53). Also, they figured out that effects of GAA obtained from *G.lucidum* on apoptosis induction and proinflammatory cytokines release in hypoxia-treated neural stem cells (NSCs) were eliminated by suppression of PI3K/AKT pathway (54). In this regard, the previous study findings demonstrated that GAA could suppress phenotypic modulation of Pulmonary Artery Smooth Muscle Cells (PASMCs) via inactivation of the PI3K/mTOR signaling pathway (55). Previous research suggested that GAA may suppress miR-17-p activity by disabling the PI3K / ACT signaling pathway. Therefore, further experimental research is still needed to analyze the underlying regulatory mechanism or GAA on miR-17-5p expression. The current study exhibited that GAA, a triterpenoid isolated from *G.lucidum*, possesses the downregulated miR-181b and miR-17-5p in Nalm-6 cells. Therefore, it can be suggested as preliminary evidence on the underlying mechanism involving the anticancer activity of GAA.

Conclusion

The present study suggested that GAA, as a natural, non-toxic compound derived from *G. lucidum* mushroom, could inhibit

the growth of human cancer Nalm-6 cells and drastically induce apoptosis. Moreover, the present study results suggested that GAA could decrease the expression of miR-17-5p and miR-181b. However, a further in-depth studies are required to elucidate the exact underlying mechanism of GAA as a novel clinical anti-cancer agent for the prevention or treatment of human leukemia.

Conflict of interest

The authors declare no conflict of interest.

References

1. Zou Y, Mei D, Yuan J, Han J, Xu J, Sun N, et al. Preparation, characterization, pharmacokinetic, and therapeutic potential of novel 6-mercaptapurine-loaded oral nanomedicines for acute lymphoblastic leukemia. *Int J Nanomedicine* 2021;16(1):1127-1141.
2. Liang C, Li Y, Wang L-N, Zhang X-L, Luo J-S, Peng C-J, et al. Up-regulated miR-155 is associated with poor prognosis in childhood acute lymphoblastic leukemia and promotes cell proliferation targeting ZNF238. *Hematology* 2021;26(1):16-25.
3. Najafi Dorcheh S, Rahgozar S, Talei D, Ghodousi E. Study on the cytotoxic effect of 10-gingerol, a derivative of ginger (*Zingiber officinale* Roscoe), on acute lymphoblastic leukemia cell lines. *J Medicinal Aromat Plants* 2021;37(1):1-12.
4. Shahverdi E, Karami P, Tavakoli F, Maki M, Moazzami M, Feizi F, et al. Treatment-related complications in childhood acute lymphoblastic leukemia: Results of medical research council UKALL X. *Middle East J Cancer* 2020;11(2):168-173.
5. Warris L, van den Heuvel-Eibrink M, Aarsen F, Pluijm S, Bierings M, Van Bos CD, et al. Hydrocortisone as an intervention for dexamethasone-induced adverse effects in pediatric patients with acute lymphoblastic leukemia: results of a double-blind, randomized controlled trial. *J Clin Oncol* 2016;34(19):2287-2293.
6. Bahmani F, Esmaeili S, Bashash D, Dehghan-Nayeri N, Mashati P, Gharehbaghian A. *Centaurea albonitens* extract enhances the therapeutic effects of Vincristine in leukemic cells by inducing apoptosis. *Biomed* 2018;99:598-607.
7. Zhao H-L, Cui S-Y, Qin Y, Liu Y-T, Cui X-Y, Hu X, et al. Prophylactic effects of sporoderm-removed *Ganoderma lucidum* spores in a rat model of streptozotocin-induced sporadic Alzheimer's disease. *J Ethnopharmacol* 2021;269:113725-113729.
8. Li C, Cui Y, Lu J, Meng L, Ma C, Liu Z, et al. Spectrum-effect relationship of immunologic activity of *Ganoderma lucidum* by UPLC-MS/MS and component knock-out method. *Food Sci Hum Wellness* 2021;10(3):278-288.
9. Tian Y-Z, Wang Z-F, Liu Y-D, Zhang G-Z, Li G. The whole-genome sequencing and analysis of a *Ganoderma lucidum* strain provide insights into the genetic basis of its high triterpene content. *Genomics* 2021;113(1 Pt 2):840-849.
10. Chang T-S, Chiang C-M, Wu J-Y, Tsai Y-L, Ting H-J. Production of a new triterpenoid disaccharide saponin from sequential glycosylation of ganoderic acid A by 2 *Bacillus* glycosyltransferases. *Biosci Biotechnol Biochem* 2021;85(3):687-690.
11. Dong Q, Li Y, Liu G, Zhang Z, Zhou H, Yang H. High oxygen treatments enhance the contents of phenolic compound and ganoderic acid, and the antioxidant and DNA damage protective activities of *Ganoderma lingzhi* fruiting body. *Front Microbiol* 2019:2363-2367.
12. Bharadwaj S, Lee KE, Dwivedi VD, Yadava U, Panwar A, Lucas S, et al. Discovery of *Ganoderma lucidum* triterpenoids as potential inhibitors against Dengue virus NS2B-NS3 protease. *Sci Rep* 2019;9(1):1-12.
13. Zhao C, Fan J, Liu Y, Guo W, Cao H, Xiao J, et al. Hepatoprotective activity of *Ganoderma lucidum* triterpenoids in

- alcohol-induced liver injury in mice, an iTRAQ-based proteomic analysis. *Food Chem* 2019;271:148-156.
14. Meng J, Wang S-z, He J-z, Zhu S, Huang B-y, Wang S-y, et al. Ganoderic acid A is the effective ingredient of Ganoderma triterpenes in retarding renal cyst development in polycystic kidney disease. *Acta Pharmacol Sin* 2020;41(6):782-790.
 15. Radwan FF, Hossain A, God JM, Leaphart N, Elvington M, Nagarkatti M, et al. Reduction of Myeloid-Derived Suppressor Cells and Lymphoma Growth by a Natural Triterpenoid. *J Cell Biochem* 2015;116(1):102-114.
 16. Wang X, Sun D, Tai J, Wang L. Ganoderic acid A inhibits proliferation and invasion, and promotes apoptosis in human hepatocellular carcinoma cells. *Mol Med Rep* 2017;16(4):3894-3900.
 17. Shao J, Li Z, Jiao G, Sun G, Zhou Z. Ganoderic acid A suppresses proliferation and invasion and induces apoptosis in human osteosarcoma cells. *Nan Fang Yi Ke Da Xue Xue Bao* 2015;35(5):619-624.
 18. Jiang J, Grieb B, Thyagarajan A, Sliva D. Ganoderic acids suppress growth and invasive behavior of breast cancer cells by modulating AP-1 and NF- κ B signaling. *Int J Mol Med* 2008;21(5):577-584.
 19. Katsaraki K, Karousi P, Artemaki PI, Scorilas A, Pappa V, Kontos CK, et al. MicroRNAs: Tiny regulators of gene expression with pivotal roles in normal B-Cell development and B-Cell chronic lymphocytic leukemia. *Cancers* 2021;13(4):593-598.
 20. Cantile M, Di Bonito M, Tracey De Bellis M, Botti G. Functional interaction among lncRNA HOTAIR and microRNAs in cancer and other human diseases. *Cancers* 2021;13(3):570-575.
 21. Vafadar A, Mokaram P, Erfani M, Yousefi Z, Farhadi A, Elham Shirazi T, et al. The effect of decitabine on the expression and methylation of the PPP1CA, BTG2, and PTEN in association with changes in miR-125b, miR-17, and miR-181b in NALM6 cell line. *J Cell Biochem* 2019;120(8):13156-13167.
 22. Grillari J, Hackl M, Grillari-Voglauer R. miR-17-92 cluster: ups and downs in cancer and aging. *Biogerontology* 2010;11(4):501-506.
 23. Ultimo S, Martelli AM, Zauli G, Vitale M, Calin GA, Neri LM. Roles and clinical implications of microRNAs in acute lymphoblastic leukemia. *J Cell Physiol* 2018;233(8):5642-5654.
 24. Fuziwara CS, Kimura ET. Insights into regulation of the miR-17-92 cluster of miRNAs in cancer. *Front Med* 2015;2:64-69.
 25. Ji J, Yamashita T, Wang XW. Wnt/beta-catenin signaling activates microRNA-181 expression in hepatocellular carcinoma. *Cell Biosci* 2011;1(1):1-8.
 26. Yan L-X, Huang X-F, Shao Q, Huang M-Y, Deng L, Wu Q-L, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *Rna* 2008;14(11):2348-2360.
 27. Tunca B, Tezcan G, Cecener G, Egeli U, Ak S, Malyer H, et al. Olea europaea leaf extract alters microRNA expression in human glioblastoma cells. *J Cancer Res Clin Oncol* 2012;138(11):1831-1844.
 28. Farzadfard E, Kalantari T, Tamaddon G. Serum expression of seven microRNAs in chronic lymphocytic leukemia patients. *J Blood Med* 2020;11:97-99.
 29. Liu J, Shi W, Wu C, Ju J, Jiang J. miR 181b as a key regulator of the oncogenic process and its clinical implications in cancer. *Biomed Rep* 2014;2(1):7-11.
 30. Zeng Z, Xiao K. Ganoderma lucidum Polysaccharide (GLP) Inhibited the progression of oral squamous cell carcinoma via the miR-188/BCL9/ β -catenin pathway. *Adv Polym Technol* 2020;2020:1-6.

31. Li A, Shuai X, Jia Z, Li H, Liang X, Su D, et al. Ganoderma lucidum polysaccharide extract inhibits hepatocellular carcinoma growth by downregulating regulatory T cells accumulation and function by inducing microRNA-125b. *J Transl Med* 2015;13(1):1-10.
32. Wu Q-P, Xie Y-Z, Deng Z, Li X-M, Yang W, Jiao C-W, et al. Ergosterol peroxide isolated from Ganoderma lucidum abolishes microRNA miR-378-mediated tumor cells on chemoresistance. *PloS one* 2012;7(8):e44579-e44583.
33. Puissegur M, Eichner R, Quelen C, Coyaud E, Mari B, Lebrigand K, et al. B-cell regulator of immunoglobulin heavy-chain transcription (Bright)/ARID3a is a direct target of the oncomir microRNA-125b in progenitor B-cells. *Leukemia* 2012;26(10):2224-2232.
34. Zhang X, Xiao C, Liu H. Ganoderic acid A protects rat H9c2 cardiomyocytes from hypoxia-induced injury via up-regulating miR-182-5p. *Cell Physiol Biochem* 2018;50(6):2086-2096.
35. Das A, Miller R, Lee P, Holden CA, Lindhorst SM, Jaboin J, et al. A novel component from citrus, ginger, and mushroom family exhibits antitumor activity on human meningioma cells through suppressing the Wnt/ β -catenin signaling pathway. *Tumour Biol* 2015;36(9):7027-7034.
36. Gonul O, Aydin HH, Kalmis E, Kayalar H, Ozkaya AB, Atay S, et al. Effects of Ganoderma lucidum (higher Basidiomycetes) extracts on the miRNA profile and telomerase activity of the MCF-7 breast cancer cell line. *Int J Med Mushrooms* 2015;17(3):231-239.
37. Zeng Z, Xiao K. Ganoderma lucidum Polysaccharide (GLP) Inhibited the progression of oral squamous cell carcinoma via the miR-188/BCL9/ β -catenin pathway. *Adv Polym Technol* 2020;2020-2025.
38. Luan C, Yang Z, Chen B. The functional role of microRNA in acute lymphoblastic leukemia: relevance for diagnosis, differential diagnosis, prognosis, and therapy. *Onco Targets* 2015;8:2903-2907.
39. Ren Y, Gao J, Liu J-Q, Wang X-W, Gu J-J, Huang H-J, et al. Differential signature of fecal microRNAs in patients with pancreatic cancer. *Mol Med Rep* 2012;6(1):201-209.
40. Nurul-Syakima A, Yoke-Kqueen C, Sabariah A, Shiran M, Singh A, Learn-Han L. Differential microRNA expression and identification of putative miRNA targets and pathways in head and neck cancers. *International Int journal ofJ molecular medicine* 2011;28(3):327-336.
41. Chen H, Chen Q, Fang M, Mi Y. microRNA-181b targets MLK2 in HL-60 cells. *Sci China Life Sci* 2010;53(1):101-106.
42. Mansueto G, Forzati F, Ferraro A, Pallante P, Bianco M, Esposito F, et al. Identification of a new pathway for tumor progression: MicroRNA-181b up-regulation and CBX7 down-regulation by HMGA1 protein. *Genes cancer* 2010;1(3):210-224.
43. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev* 2006;6(11):857-866.
44. Aggarwal BB, Kunnnumakkara AB, Harikumar KB, Gupta SR, Tharakan ST, Koca C, et al. Signal transducer and activator of transcription-3, inflammation, and cancer: how intimate is the relationship? *Ann N Y Acad Sci* 2009;1171(1):59-76.
45. Yao X, Li G, Xu H, Lü C. Inhibition of the JAK-STAT3 signaling pathway by ganoderic acid A enhances chemosensitivity of HepG2 cells to cisplatin. *Planta Med* 2012;78(16):1740-1748.
46. Yang Y, Zhou H, Liu W, Wu J, Yue X, Wang J, et al. Ganoderic acid A exerts antitumor activity against MDA MB 231 human breast cancer cells by inhibiting the Janus kinase 2/signal transducer and activator of transcription 3 signaling pathway. *Oncol* 2018;16(5):6515-6521.

47. Gill BS, Kumar S. Evaluating anti-oxidant potential of ganoderic acid A in STAT 3 pathway in prostate cancer. *Mol Biol Rep* 2016;43(12):1411-1422.
48. Liu F, Zhang F, Li X, Liu Q, Liu W, Song P, et al. Prognostic role of miR-17-92 family in human cancers: evaluation of multiple prognostic outcomes. *Oncotarget* 2017;8(40):69125-69128.
49. Mu P, Han Y-C, Betel D, Yao E, Squatrito M, Ogrodowski P, et al. Genetic dissection of the miR-17~ 92 cluster of microRNAs in Myc-induced B-cell lymphomas. *Genes Dev* 2009;23(24):2806-2811.
50. Karar J, Maity A. PI3K/AKT/mTOR pathway in angiogenesis. *Front Mol Neurosci* 2011;4:51-59.
51. Noorolyai S, Shajari N, Baghbani E, Sadreddini S, Baradaran B. The relation between PI3K/AKT signalling pathway and cancer. *Gene* 2019;698:120-128.
52. Cheng Y, Xie P. Ganoderic acid A holds promising cytotoxicity on human glioblastoma mediated by incurring apoptosis and autophagy and inactivating PI3K/AKT signaling pathway. *J Biochem Mol Toxicol* 2019;33(11):e22392-22398.
53. Chang Y, Kong R. Retracted: Ganoderic acid A alleviates hypoxia-induced apoptosis, autophagy, and inflammation in rat neural stem cells through the PI3K/AKT/mTOR pathways. *Phytother Res* 2019;33(5):1448-1456.
54. Meng Y, Ning Q, Liu Y, Pang Y, Ren H, Yang T, et al. Ganoderic Acid A suppresses the phenotypic modulation of pulmonary artery smooth muscle cells through the inactivation of PI3K/Akt pathway in pulmonary arterial hypertension. *Food Sci Technol* 2021;1-9